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Is Allantoin in Serum and Urine a Useful Indicator of Exercise-Induced Oxidative Stress in Humans?

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To assess whether allantoin levels in serum and urine are influenced by exhaustive and moderate exercise and whether allantoin is a useful indicator of exercise-induced oxidative stress in humans, we made subjects perform exhaustive and moderate (100% and 40% VO_2max) cycling exercise and examined the levels of allantoin, thiobarbituric acid reactive substances (TBARS) and urate in serum and urine. Immediately after exercise at 100% VO_2max, the serum allantoin/urate ratio was significantly elevated compared with the resting levels while the serum urate levels was significantly elevated 30 min after exercise. The serum TBARS levels did not increase significantly compared with the resting levels. Urinary allantoin excretion significantly increased during 60 min of recovery after exercise, however, urinary urate excretion decreased significantly during the same period. The urinary allantoin/urate ratio also rapidly increased during 60 min of recovery after exercise. Urinary TBARS excretion decreased during the first 60 min of the recovery period and thereafter significantly increased during the latter half of the recovery period. On the contrary, after 40% VO_2max of exercise, no significant changes in the levels of urate, allantoin and TBARS in serum or urine were observed. These findings suggest that allantoin levels in serum and urine may reflect the extent of oxidative stress *in vivo* and that the allantoin which appeared following exercise may have originated not from urate formed as a result of exercise but from urate that previously existed in the body. Furthermore, these findings support the view that allantoin in serum and urine is a more sensitive and reliable indicator of *in vivo* oxidative stress than lipid peroxidation products measured as TBARS.

Keywords: Allantoin, urate, TBARS, exercise, oxidative stress

INTRODUCTION

It has been demonstrated that exhaustive exercise results in oxidative damage to cells and tissues by inducing the formation of free radicals. Davies *et al.*[1] showed that free radical formation occurs in liver and skeletal muscle during exercise as using electron spin resonance. However,

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it is very difficult to detect the *in vivo* formation of free radicals directly, so most investigators studying oxidative stress in exercise have measured not free radicals but lipid peroxidation products, which are produced through oxidation of lipids by free radicals. The lipid peroxidation products which have been previously measured as an indicator of oxidative stress were pentane in expired air,[2,3] low-density lipoprotein diene conjugates[4,5] and thiobarbituric acid reactive substances (TBARS).[2,6–9] Among these, TBARS values are most widely used because the method of determination of this indicator is easy and sensitive. However, the substances that react with thiobarbituric acid (TBA) are not unique to serum and plasma, so it is doubted that the measured TBARS values really reflect the amount of lipid peroxidation products *in vivo*.[10] In fact, studies by various investigators examining TBARS in exercise have not yielded consistent results. Kanter *et al.*[2] reported that levels of expired pentane and serum TBARS were increased by treadmill exercise at 90% VO_2max. Lovlin *et al.*[6] found a 26% increase in plasma TBARS induced by maximal exercise at 100% VO_2max. Sumida *et al.*[7] found also that acute exhaustive exercise resulted in a slight, but significant increase in serum TBARS concentration. On the other hand, Viinkka *et al.*[8] found that serum lipid peroxidation products did not change during maximal cycling exercise. Rokitzki *et al.*[9] reported that serum TBARS concentration decreased after a marathon race.

Several substances have been measured as indicators of oxidative stress *in vivo* in place of TBARS. Allantoin is one of them. Humans lack uricase, which oxidizes urate to allantoin, but allantoin exists in human body fluid because it is produced through non-enzymatic oxidation of urate by free radicals.[11,12] With respect to the relationship between allantoin levels and oxidative stress, it has been reported that plasma allantoin levels in patients with rheumatism[12] or Wilson's disease,[13] which are diseases associated with chronic oxidative stress, are elevated compared with those in healthy controls. The effect of exercise on allantoin levels has been reported only by Hellsten *et al.*[14] who demonstrated a marked increase of allantoin levels in plasma and skeletal muscle after exhaustive cycling exercise. However, the relationship between allantoin formation in exercise and the intensity of exercise, and the relationship between allantoin formation and lipid peroxidation in exercise are not known yet. Moreover, the influence of exercise on urinary allantoin excretion is not known at all.

In the present study, to assess whether allantoin levels in serum and urine are influenced by exhaustive or moderate exercise and whether allantoin levels serve as a useful indicator of exercise-induced oxidative stress in humans, we made the subjects perform two kinds of exercise (exhaustive and moderate) and examined the levels of allantoin, TBARS and urate in serum and urine.

METHODS

Experimental Protocol

We performed two kinds of experiments as follows. In experiment 1, we examined the correlation between urate levels and allantoin levels *in vivo* by measuring the levels of urate and allantoin in serum. Forty-four healthy male volunteers, 20–30 years of age, took part. They did not participate regularly in physical activity. Venous blood samples were obtained from the subjects at 9:00–11:00 a.m. after 12 h of fasting. None of the participants for the present study had any symptoms of infection at that time. Serum was separated by centrifugation at 3000*g* for 20 min, transferred to a fresh tube and stored at −80°C until analysis.

In experiment 2, to investigate whether a difference in exercise intensity influences allantoin, urate and TBARS levels in serum and urine, we made seven healthy male subjects perform exhaustive or moderate cycling exercise. The subjects, who were players of track and field

throwing events, performed intense exercise training including weight training daily and participated in competitive games. Their mean height and mean weight were 181.3 ± 2.4 cm and 103.0 ± 8.0 kg, respectively. The maximal oxygen uptake per kg body weight of the subjects was 61.6 ± 7.1 ml/min. The subjects were fully informed of the potential risks and discomfort associated with the experiment before giving informed consent to participate. The study was approved by the Ethics Committee of Nippon Medical School.

The subjects were instructed not to perform strenuous exercise on the day before the experiments. Breakfast, lunch and supper were provided on the day before exercise and breakfast was provided on the day of exercise so that the intake of antioxidants such as vitamin C, vitamin E and β-carotene was not different between the two experiments. The contents of the above provided food were as follows: Breakfast: calorie intake, 1524 kcal; carbohydrate, 200 g; fat, 47 g; protein, 68 g; vitamin C, 78 mg; vitamin E, 3.5 mg; β-carotene, 195 μg. Lunch: calorie intake, 1549 kcal; carbohydrate, 184 g; fat, 51 g; protein, 74 g; vitamin C, 74 mg; vitamin E, 3.7 mg; β-carotene, 285 μg. Supper: calorie intake, 1851 kcal; carbohydrate, 231 g; fat, 66 g; protein, 80 g; vitamin C, 160 mg; vitamin E, 7.0 mg; β-carotene, 2885 μg.

On the day of the experiment, the subjects came to the laboratory at 8:00 a.m., had the breakfast provided, did a complete bladder emptying, and remained at rest until starting the exercise. After resting for 2 h, blood and urine were collected, and exercise was performed at 100% VO_2max or 40% VO_2max. In the case of exercise performed at 100% VO_2max (100% exercise), the subjects performed about 10 min of cycling exercise to exhaustion by an incremental workload method. In the case of exercise performed at 40% VO_2max (40% exercise), the subjects performed about 40 min of cycling exercise with an energy consumption equal to the 100% exercise. After the completion of the exercise, the subjects remained at rest for a 4-h recovery period. In the recovery period, blood and urine were collected at 30, 60, 120, 180 and 240 min after the completion of exercise. Blood samples were immediately centrifuged, and the serum and urine were stored at −80°C until analysis. The two exercise experiments, 100% exercise and 40% exercise, were separated by at least a one-week interval.

Analysis

Allantoin levels in serum and urine were determined by high performance liquid chromatography (HPLC) according to the modified method of Grootveld *et al.*[12] and Lagendijk *et al.*[11] An outline of the method of measurement is provided as follows.

One hundred μl of deproteinized plasma obtained by passing the plasma through an Amicon Microcon-3 filter (molecular weight cut-off 3000) were injected into the HPLC equipped with two Inertosil ODS-3 columns (GL Sciences, Tokyo, Japan). The fractions with retention times in the range of 6.6–7.8 min, at which the allantoin standard was known to be eluted, were collected and evaporated to dryness using a vacuum concentrator (Sakuma Fractory, Tokyo, Japan). The residue was redissolved in 200 μl of 0.12 M NaOH and heated at 100°C for 20 min. After adding 200 μl of 1.0 M HCl, the mixture was heated at 100°C for 5 min. Thereafter, 20 μl of 3.0 mM 2,4-dinitrophenylhydrazine (2,4-DNPH) in 1.0 M HCl was added and the mixture was heated at 100°C for 5 min. In these reactions, allantoin was finally converted into glyoxylate-2,4-dinitrophenylhydrazone (G-2,4-DNPH). Fifty μl of the final solution was injected into an HPLC equipped with a NovaPack C18 column (Waters, Tokyo, Japan) and G-2,4-DNPH was detected by monitoring the absorbance at 360 nm.

The levels of lipid peroxidation products in serum and urine were determined as TBARS according to a HPLC method of Kosugi *et al.*[15] In this method, serum or urine heated with TBA under acidic conditions were injected into an

HPLC equipped with a YMC Pack A-303 column (YMC, Kyoto, Japan) and the eluted substances were detected at 532 nm.

Urate levels in serum and urine were determined using the Uric Acid B-Test Wako kit (Wako Pure Chemical Industries, Tokyo, Japan). The creatinine concentration in urine was measured by means of the Creatinine Test Wako kit (Wako Pure Chemical Industries, Tokyo, Japan).

The haematocrit was measured by microcapillary method. The exercise with large quantities of perspiration results in overestimating the levels of plasma parameters because of the plasma volume depletion. Exhaustive and prolonged exercises led to 13.0% and 7.1% of the decrease in plasma volume, respectively.[16] In the present study, the changes in plasma volume by exercise were determined from the changes of the hematocrit using the formula described by Van Beaumont *et al.*[17] The decreases of the plasma volume in 100% and 40% exercise were about 10% and 15%, respectively. All serum parameters were corrected for the changes of the plasma volume. To eliminate the influence of exercise on glomerular filtration rate, the values for urinary excretion were corrected against urinary creatinine excretion and expressed as per mg of creatinine.

Statistical Analysis

All values are presented as mean ± SEM. Statistical significance of changes in parameters during exercise and recovery was tested by analysis of variance (ANOVA) with repeated measure design. Statistical significance was accepted if $p < 0.05$.

RESULTS

Reliability of Allantoin Analysis

In the present study, allantoin was converted to glyoxylic acid, reacted with 2,4-DNPH and measured as G-2,4-DNPH. The plasma urate level in normal males is 300 μM while the plasma allantoin level in normal males is about 15 μM, that is, 1/20 the urate level.[12] Urate is dehydrated and reacts with 2,4-DNPH in the same way as allantoin, so contamination of a little urate in the allantoin fraction collected in the first HPLC analysis can cause a markedly high overestimation of the serum allantoin level. Glyoxylate is also present in human plasma and will overestimate the plasma allantoin levels if not removed. Therefore, the first HPLC analysis was performed to collect the fractions which did not contain glyoxylate and urate. A typical chromatogram obtained in the first HPLC analysis is shown in Figure 1A. The retention times for allantoin, glyoxylate and urate were 7.2, 8.4 and 10.0 min, respectively.

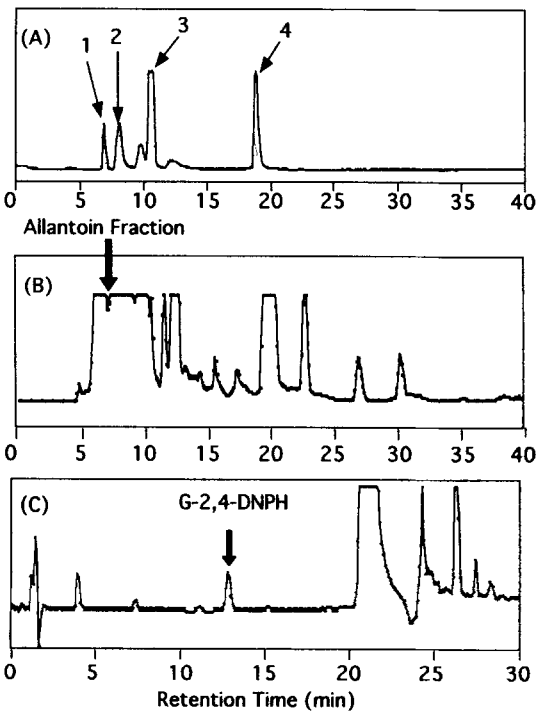

FIGURE 1 Typical chromatogram of HPLC for allantoin analysis. (A) Chromatogram of a standard mixture with detection at 210 nm in the first HPLC analysis. 1, allantoin; 2, glyoxylic acid; 3, parabanic acid; 4, uric acid. (B) Chromatogram of deproteinized serum in the first HPLC analysis. (C) Chromatogram of G-2,4-DNPH derived from a serum sample with detection at 360 nm in the second HPLC analysis.

Furthermore, when the collected allantoin fraction was applied to the first HPLC analysis again, no peak appeared in the position of glyoxylate and urate, so it was unlikely that any glyoxylate and urate contaminated the allantoin fraction.

Urate, Allantoin, Allantoin/Urate Ratio and TBARS in Serum

The serum urate concentration, allantoin concentration, allantoin/urate ratio in the subjects in experiment 1 (healthy controls) and the subjects in experiment 2 (athletes) are presented in Table I. The pre-exercise values are indicated as the values of the subjects in experiment 2. Each of these values was significantly elevated in the case of the athletes compared with the healthy controls ($p < 0.05$).

We investigated the correlation between serum urate levels and serum allantoin levels in the healthy controls in experiment 1 (Figure 2). No significant correlation was observed.

The changes in serum urate levels in experiment 2 are shown in Figure 3A. In the case of 100% exercise, serum urate levels were significantly increased at 30 min after exercise compared with the resting level ($p < 0.05$), reaching a peak level at 60 min after exercise, and the levels remained significantly high throughout the recovery period. On the contrary, no significant change in serum urate levels was observed in the case of 40% exercise (Figure 3A). Serum allantoin levels in the case of 100% exercise started to increase immediately after exercise and remained significantly high during 60 mim of recovery ($p < 0.05$) (Figure 3B). However, serum allantoin levels in the case of 40% exercise did not significantly change throughout the recovery period (Figure 3B). The serum allantoin/urate ratio in the case of 100% exercise was significantly elevated during 60 min of recovery period ($p < 0.05$) whereas in the case of 40% exercise the ratio did not change significantly (Figure 3C). Serum TBARS levels in the case of 100% or 40% exercise did not significantly change compared with the resting level (Figure 3D).

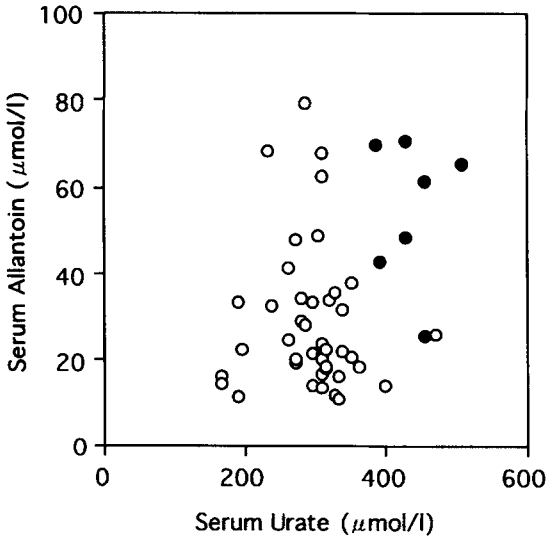

FIGURE 2 Correlation between serum urate levels and serum allantoin levels in healthy controls ($n = 44$) and athletes ($n = 7$). ○ The levels of the subjects in the experiment 1 (healthy controls). ● The pre-exercise levels of the subjects in the experiment 2 (athletes). All values are the resting values of the subjects.

TABLE I Concentration of serum urate, serum allantoin and serum allantoin/urate ratio in healthy controls and athletes. The pre-exercise values are indicated as the values of the athletes

	Healthy control ($n = 44$)		Athletes ($n = 7$)	
	Mean ± SEM	Range	Mean ± SEM	Range
Serum urate (μmol/l)	298.9 ± 9.4	166.6–470.0	425.1 ± 13.1*	319.7–507.7
Serum allantoin (μmol/l)	28.0 ± 2.2	11.1–68.2	65.0 ± 8.8*	37.1–91.2
Allantoin/urate (%)	4.6 ± 0.4	1.5–13.2	14.4 ± 2.6*	7.1–19.5

*$p < 0.05$. Significantly different vs healthy control.

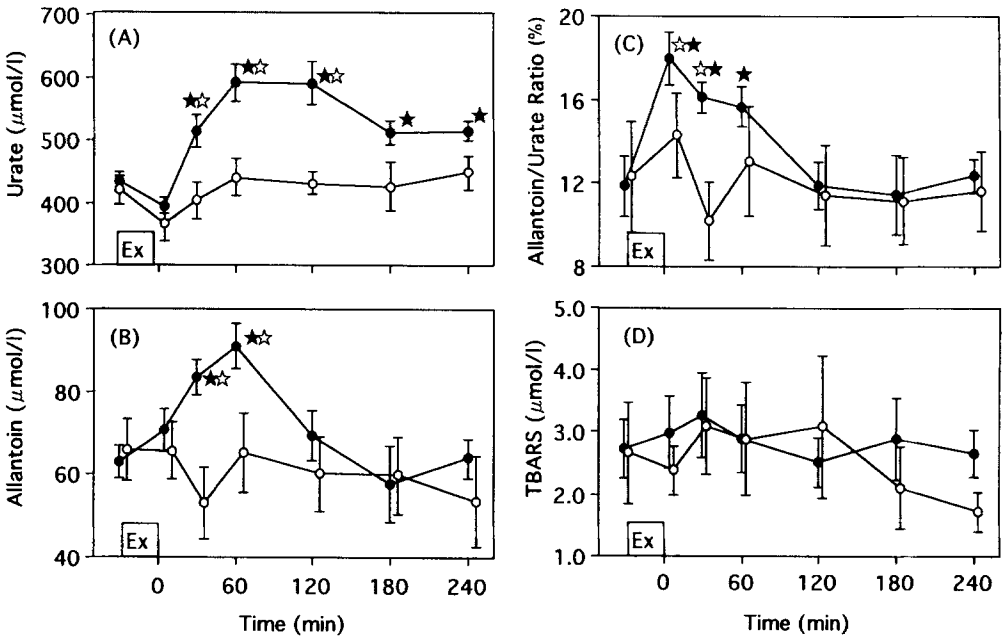

FIGURE 3 Serum urate concentration (A), serum allantoin concentration (B), serum urate/allantoin ratio (C) and serum TBARS concentration (D) after exercise at 100% VO_2max (○) and 40% VO_2max (●). Values are presented as mean ± SEM ($n = 7$). ★ $p < 0.05$, significant difference from the resting level, ☆ $p < 0.05$, significant difference between 100% exercise and 40% exercise.

Urinary Excretion of Urate, Allantoin, TBARS, Creatinine and Allantoin/Urate Ratio

In the case of 100% exercise, urinary urate excretion significantly decreased ($p < 0.05$) from the resting level during 60 min of recovery and then significantly increased after 120 min of recovery ($p < 0.05$) (Figure 4A). Urinary allantoin excretion significantly increased immediately after exercise ($p < 0.05$) and remained at a high level during 240 min of recovery (Figure 4B). The urinary allantoin/urate ratio also significantly increased during the first 30 min of recovery and returned to the resting level thereafter (Figure 4C). Urinary TBARS excretion decreased during the first 60 min of recovery and significantly increased slowly during the latter half of the recovery period (Figure 4D). Urinary creatinine excretion decreased immediately after exercise ($p < 0.05$) and returned to the resting level thereafter (Figure 4E).

On the contrary, no significant changes in urinary excretion of allantoin, urate and TBARS, and allantoin/urate ratio were observed in the case of 40% exercise (Figure 4A–D). However, urinary creatinine excretion significantly increased during the recovery of 40% exercise (Figure 4E).

DISCUSSION

Allantoin originates from urate, so there is a possibility that an increase in urate levels may influence allantoin levels independent of oxidative stress *in vivo*. To examine this possibility, we investigated the correlation between serum urate and allantoin levels in normal healthy males in the resting period. No significant correlation was observed (Figure 1). This observation indicates that the levels of allantoin is not influenced by the amount of urate in the absence of oxidative stress. However, the pre-exercise levels of serum urate, allantoin and allantoin/urate ratio in the athletes

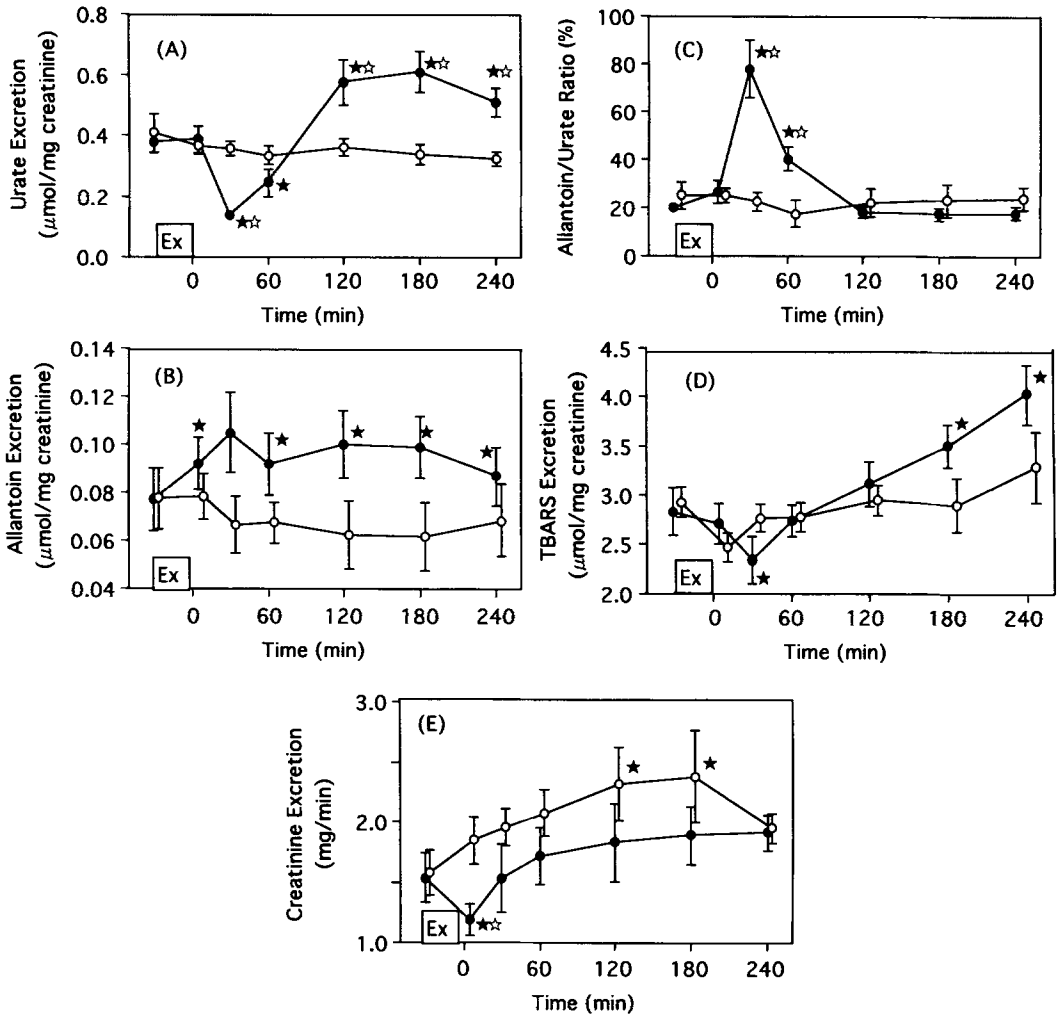

FIGURE 4 Urinary excretion of urate (A), allantoin (B), TBARS (D), creatinine (E) and urinary allantoin/urate ratio (C) after exercise at 100% VO_2max (○) and 40% VO_2max (●). Urinary excretion of urate, allantoin and TBARS are standardized based on the amount of creatinine excretion. Values are presented as mean ± SEM. ★ $p < 0.05$, significant difference from the resting level, ☆ $p < 0.05$, significant difference between 100% exercise and 40% exercise.

(the subjects in experiment 2) were significantly elevated compared with the control (the subjects in experiment 1) (Table I). As mentioned in the results section, it seems unlikely that any urate would contaminate the allantoin fraction (Figure 1). The high serum levels of urate, allantoin and allantoin/urate ratio in athletes may be a characteristic of athletes who perform intensive physical training every day. Two recent studies have demonstrated that high intensity training leads to the decreased resting levels of adenine nucleotides in human skeletal muscle and that a part of purines lost from skeletal muscle is in turn oxidized to hypoxanthine, xanthine and uric acid.[18,19] Therefore, we speculate that athletes may repeat the degradation of adenine nucleotides and the oxidation of urate in skeletal muscle as a result of high intensity training, resulting in elevated levels of serum urate, allantoin levels and allantoin/urate ratio.

In the present study, it was found that exhaustive cycling exercise at 100% VO_2max led to a

marked increase in serum allantoin levels whereas moderate exercise at 40% VO_2max did not. This observation suggests that allantoin formation during exercise may be influenced by intensity of exercise, in other words, the extent of oxidative stress induced by exercise. As mentioned in introduction, it has reported that plasma allantoin levels increase in patients with rheumatism[12] or Wilson's disease[13] and the subjects who performed intense exercise.[14] Therefore, taken together with these previous observations, the present findings suggest that serum allantoin levels may be influenced by the extent of *in vivo* oxidative stress due to exercise and that the plasma or serum allantoin level may be a sensitive indicator of oxidative stress *in vivo*.

In this study, serum allantoin levels increased immediately after exercise, especially allantoin/urate ratio significantly increased immediately after exhaustive exercise, while serum urate elevated after 30 min of recovery. The mechanism responsible for the increase in urate levels after exercise is proposed to be as follows. Exhaustive exercise induces degradation of adenine nucleotides in skeletal muscle and produces hypoxanthine. Hypoxanthine is released into blood, taken into the liver and oxidized to urate by xanthine oxidase, and the urate is released into the blood.[20] Thus, there is a delay of several minutes in the appearance of increased urate levels in blood after exercise.[14] The present observation, which showed that the allantoin/urate ratio increased immediately after exercise, suggests that allantoin increased immediately after exercise may originate not from urate formed due to exercise but from urate that previously existed in the body. Furthermore, the amount of urate present *in vivo* may influence antioxidant capacity in scavenging free radicals. In fact, we have found that the amount of urate present in the body prior to exercise influences the amount of urinary lipid peroxidation products excreted after exercise (submitted for publication).

The previous studies on urinary allantoin excretion have been not concerning oxidative stress in exercise but rather nutrient assessment in domestic animals, so the present study is the first report showing that a change in urinary excretion of allantoin occurs as a result of exercise. Urinary allantoin excretion significantly increased during 240 min of recovery (Figure 4B) whereas urinary urate excretion decreased during 60 min of recovery (Figure 4A). Urinary urate excretion in the kidney is regulated by a mechanism called the four component system. This system consists of glomerular filtration, tubular reabsorption, tubular secretion and post-secretory reabsorption for urate.[21,22] The decrease in urinary urate excretion during the early period of recovery after exercise is caused by an increase in substances which compete with urate for secretion in the renal tubules, especially lactate.[23] As far as we know, renal handling of allantoin has been characterized only in rats. In rats, allantoin is filtered at the glomerulus and neither reabsorbed nor secreted along the nephron.[24,25] So, allantoin clearance is essentially identical with inulin clearance.[24,25] The features of renal handling of allantoin in humans are not known, however, it is likely that allantoin may be regulated by the same mechanism as that in rats, because urinary allantoin excretion was not inhibited but increased after exhaustive exercise. When the depression of the urinary creatinine excretion (Figure 4E), which relates to the glomerular filtration rate, immediately after exhaustive exercise is taken into consideration, the increase of urinary allantoin excretion immediately after exercise may reflect the marked increase of allantoin production during exercise. The ratio of allantoin/urate in urine significantly increased immediately after exhaustive exercise, however, this increase might be induced by a decrease of urate excretion. In the future, further examination of the regulatory mechanisms controlling allantoin levels in humans is necessary.

In the present study, though allantoin and TBARS were both used as indicators of oxidative stress, the changes in these indicators after exercise were different. In other words,

exhaustive exercise led to increased serum allantoin levels but moderate exercise did not, and neither exhaustive nor moderate exercise led to a significant change in serum TBARS levels compared with the resting level. A similar difference between allantoin and TBARS levels has been reported by Ogihara *et al.*,[13] who showed that patients with Wilson's disease had normal plasma TBARS levels in spite of high plasma allantoin levels and suggested that allantoin levels reflect oxidative damage in the intracellular aqueous compartment while TBARS reflect such damage in the intracellular lipid compartment. In addition, the substances that react with TBA are not unique to serum and plasma samples, so it is doubted that the measured TBARS values in serum or plasma really reflect the amount of lipid peroxidation products present *in vivo*.[10] Therefore, these findings suggest that the serum allantoin level may be a more sensitive and reliable indicator of *in vivo* oxidative stress in exercise than serum TBARS levels.

In the case of 100% exercise, urinary TBARS excretion increased slowly during the latter half of the recovery period (Figure 4D). With respect to delayed appearance of lipid peroxidation products after exercise, it has reported that serum TBARS increased several hours after exercise and this increase corresponded to the increase of lactate dehydrogenase activity and creatine phosphokinase activity in serum.[26] However, malonaldehyde is derived from not only lipid peroxidation products but also 2-deoxyribose,[27,28] so TBARS does not always reflect lipid peroxidation products. Therefore, the present observation did not prove whether the increase of urinary TBARS excretion during the latter half of the recovery really reflect lipid peroxidation *in vivo* or not. More detailed investigation about urinary TBARS excretion in exercise is necessary.

In conclusion, to assess whether the change in allantoin levels actually reflects the extent of oxidative stress *in vivo*, we made healthy male subjects perform exhaustive or moderate cycling exercise. Exhaustive cycling exercise led to a significant increase in serum allantoin/urate ratio immediately after exercise prior to the increase in serum urate, while moderate exercise did not. Serum TBARS levels did not significantly change in the case of exhaustive or moderate exercise. Urinary allantoin excretion and the urinary allantoin/urate ratio significantly increased immediately after exercise. These findings suggest that allantoin levels in serum and urine may reflect the extent of oxidative stress *in vivo* and that the allantoin appeared immediately after exercise may originate from urate which existed in the body before exercise. Further, these findings support the view that the serum allantoin level and urinary allantoin excretion may be more sensitive indicators of oxidative stress *in vivo* than lipid peroxidation products measured as TBARS.

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